

# ASSAY OF POLY- $\beta$ -HYDROXYBUTYRIC ACID

JOHN H. LAW AND RALPH A. SLEPECKY

*Department of Chemistry, Harvard University, Cambridge, Massachusetts, and Department of Biological Sciences, Northwestern University, Evanston, Illinois*

Received for publication December 31, 1960

## ABSTRACT

LAW, JOHN H. (Harvard University, Cambridge, Mass.) AND RALPH A. SLEPECKY. Assay of poly- $\beta$ -hydroxybutyric acid. *J. Bacteriol.* **82**:33-36. 1961—A convenient spectrophotometric assay of bacterial poly- $\beta$ -hydroxybutyric acid has been devised. Quantitative conversion of poly- $\beta$ -hydroxybutyric acid to crotonic acid by heating in concentrated sulfuric acid and determination of the ultraviolet absorption of the produce permits an accurate determination of this material in quantities down to 5  $\mu$ g. This method has been used to follow the production of poly- $\beta$ -hydroxybutyric acid by *Bacillus megaterium* strain KM.

Recent reports concerning the widespread occurrence of poly- $\beta$ -hydroxybutyric acid (Forsyth, Haward, and Roberts, 1958; Haward, Forsyth, and Roberts, 1959; Haward, 1959; Morris and Roberts, 1959; Levine and Wolochow, 1960; Kallio and Harrington, 1960) and of its metabolic significance (Weibull, 1953; Doudoroff and Stanier, 1959; Stanier et al., 1959; Wilkinson, 1959) have stimulated a renewed interest in this substance, first described many years ago by Lemoigne (1926). In the course of metabolic investigations of this substance a convenient and reliable spectrophotometric assay for poly- $\beta$ -hydroxybutyric acid has been developed (Slepecky and Law, 1960a). Because it is expected that this assay will prove useful in further experiments with this material, the details of the procedure will be discussed.

A common method for estimating poly- $\beta$ -hydroxybutyric acid is a gravimetric procedure based upon the fact that this polymer is soluble only in boiling chloroform, so that all contaminants may be removed by extraction with other solvents (Lemoigne, 1926). This method, of course, requires that milligrams of polymer be present for a convenient determination. Furthermore, it will be shown that this method does

not give a precise measure of poly- $\beta$ -hydroxybutyric acid unless the polymer can be reprecipitated quantitatively.

A more sensitive method has been devised by Williamson and Wilkinson (1958). In this procedure the turbidity of the lipid granules is measured subsequent to complete dissolution of the cells in a sodium hypochlorite solution. The method is standardized by comparison to the gravimetric method. It suffers from the need for careful standardization for each organism studied and from the fact that only polymer in the native lipid granules can be measured.

The method described here is a simple and specific one that can be used to measure poly- $\beta$ -hydroxybutyrate in any form. The principle of this method lies in two observations: first, that poly- $\beta$ -hydroxybutyrate can be converted quantitatively to crotonic acid by heating in concentrated sulfuric acid, and second, that the ultraviolet absorption maximum of crotonic acid is shifted to 235  $m\mu$  when concentrated sulfuric acid is the solvent (Slepecky and Law, 1960a).

## MATERIALS AND METHODS

Reagent grade solvents and sulfuric acid were used in all experiments. Ultraviolet absorption measurements were made in a spectrophotometer using standard silica cells.

*Bacillus megaterium* strain KM was grown in a Biogen (American Sterilizer Company, Erie, Pa.) using the medium of Macrae and Wilkinson (1958). Forty liters of medium yielded 543 g wet weight, of cells. The polymer granules were isolated from 100 g wet weight of cells by the method of Williamson and Wilkinson (1958). The granules were washed thoroughly with acetone, alcohol, and ether. The residue was dried and the polymer was extracted into boiling chloroform. After filtration through a Celite pad, the polymer was reprecipitated by addition of the chloroform solution to 5 volumes of acetone

and cooling to  $-20^{\circ}\text{C}$ . The yield of material was 14.2 g, mp  $173.5$  to  $175.5^{\circ}\text{C}$ .

Analysis of polymer:



Calculated: C 55.80, H 7.03

Found: C 55.01, H 7.67

This material was used as a standard for the poly- $\beta$ -hydroxybutyric acid assay.

For the assay of polymer in small quantities of cells, the organisms were centrifuged in polypropylene centrifuge tubes (Ivan Sorvall, Inc., Norwalk, Conn.; no. 254) which had been previously washed thoroughly with ethanol and hot chloroform to remove plasticizers. The cell paste was resuspended in a volume of commercial sodium hypochlorite solution (Clorox or equivalent) equal to the original volume of medium. After 1 hr at  $37^{\circ}\text{C}$  the lipid granules were centrifuged, washed with water, and then washed with acetone and alcohol. Finally, the polymer was dissolved by extraction with three small portions of boiling chloroform, the chloroform solution was filtered, and the filtrate was used for poly- $\beta$ -hydroxybutyrate assay. In cases where considerable polymer was present the acetone and alcohol washings were unnecessary, but they served to remove water which interferes with the extraction of the polymer into chloroform.

For the spectrophotometric assay of polymer, a sample containing 5 to  $50\text{ }\mu\text{g}$  polymer in chloroform is transferred to a clean test tube. The

chloroform is evaporated and 10 ml of concentrated  $\text{H}_2\text{SO}_4$  are added, the tube is capped with a glass marble and heated for 10 min at  $100^{\circ}\text{C}$  in a water bath. The solution is cooled, and, after thorough mixing, a sample is transferred to a silica cuvette and the absorbance at  $235\text{ m}\mu$  is measured against a sulfuric acid blank. The amount of crotonic acid is calculated from the molar extinction coefficient, which is  $1.55 \times 10^4$  (Slepecky and Law, 1960b).

## RESULTS

Figure 1 shows the linear relationship between polymer concentration and absorbance at  $235\text{ m}\mu$ . This determination was made with the purified polymer sample described above. The slope of this curve may be used to calculate a molar extinction coefficient of  $1.56 \times 10^4$  (using a molecular weight of 186 for the "depolymerized" poly- $\beta$ -hydroxybutyric acid) which agrees, within experimental error, with that of crotonic acid (Slepecky and Law, 1960b).

To compare this method of polymer assay with the widely employed gravimetric method, the growth of a culture of *B. megaterium* was followed by withdrawal of 50-ml samples (12 liters, total culture volume) for assay of polymer by both methods. The results of this experiment are shown in Fig. 2.

## DISCUSSION

The assay method for poly- $\beta$ -hydroxybutyric acid has been used to estimate polymer extracted from various organisms and under a variety of conditions (Slepecky and Law, 1960a). It has proved reliable and convenient in all instances. Certain materials, notably carbohydrates, cause some interference with this assay (Slepecky and Law, 1960b). In addition, with some cells which do not contain polymer, an insoluble material is obtained after hypochlorite treatment which contains an interfering substance. This material gives a spectrum in sulfuric acid quite different from the typical crotonate spectrum (Fig. 3). The nature of this material is not known. However, it is important to check carefully the entire spectrum from  $220$  to  $260\text{ m}\mu$  when using this assay method with an unknown sample.

The comparison of the gravimetric assay method with the spectrophotometric method for determination of poly- $\beta$ -hydroxybutyric acid in crude samples reveals that the former gives values about 10% higher than the latter. This is prob-

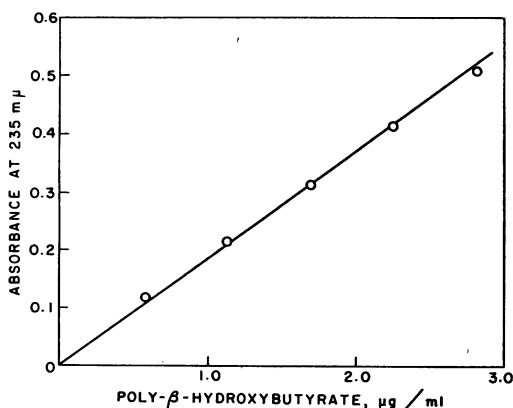


FIG. 1. Relationship between poly- $\beta$ -hydroxybutyric acid concentration and absorbance at  $235\text{ m}\mu$ . Samples of poly- $\beta$ -hydroxybutyric acid were heated in concentrated  $\text{H}_2\text{SO}_4$  for 10 min before absorbance was measured.

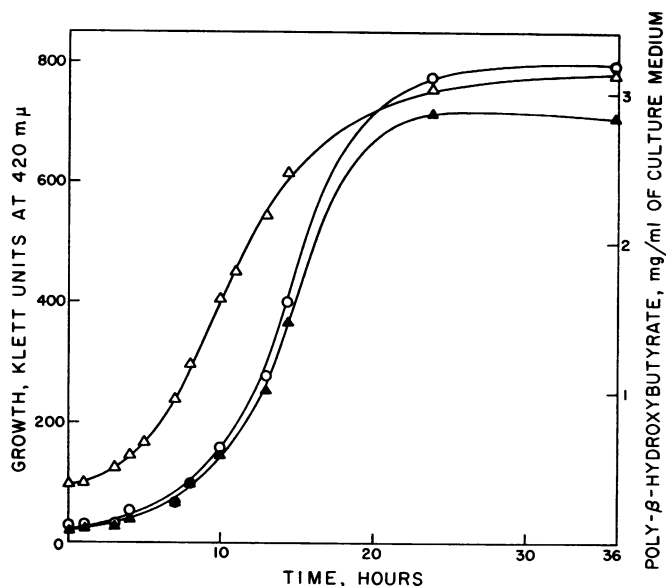


FIG. 2. Poly- $\beta$ -hydroxybutyric acid formation by *Bacillus megaterium* strain KM.  $\triangle$ — $\triangle$ , Growth;  $\circ$ — $\circ$ , gravimetric polymer assay;  $\blacktriangle$ — $\blacktriangle$ , spectrophotometric polymer assay. Medium of Macrae and Wilkinson (1958).

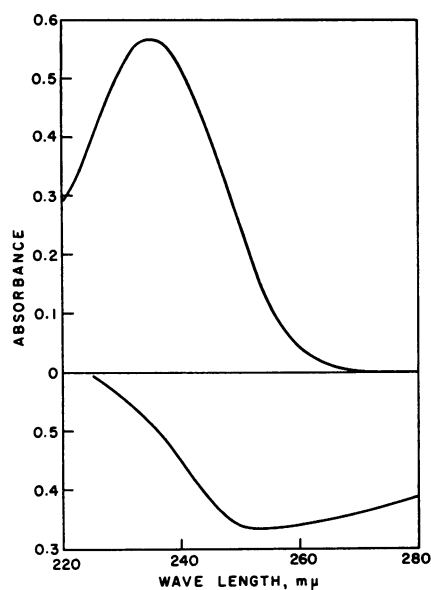


FIG. 3. Spectra of crotonic acid and of an unknown interfering substance. Upper curve—poly- $\beta$ -hydroxybutyric acid after heating in concentrated  $H_2SO_4$ . Lower curve—interfering substance extracted from cells of *Bacillus megaterium* lacking poly- $\beta$ -hydroxybutyric acid, after heating in concentrated  $H_2SO_4$ .

ably due to chloroform-soluble impurities in the crude polymer which were not removed by acetone and alcohol washing of the native lipid particles. Reprecipitated polymer, on the other hand, gives the theoretical amount of crotonic acid on sulfuric acid treatment.

#### ACKNOWLEDGMENTS

This work was supported by grants from the National Science Foundation (G10502; G7597) and the U. S. Public Health Service (RG7087).

#### LITERATURE CITED

- DOUDOROFF, M., AND R. Y. STANIER. 1959. Role of poly- $\beta$ -hydroxybutyric acid in the assimilation of organic carbon by bacteria. *Nature* **183**:1440-1442.
- FORSYTH, W. G. C., A. C. HAWARD, AND J. B. ROBERTS. 1958. Occurrence of poly- $\beta$ -hydroxybutyric acid in aerobic gram-negative bacteria. *Nature* **182**:800-801.
- HAWARD, A. C. 1959. Poly- $\beta$ -hydroxybutyrate inclusions in the classification of aerobic gram-negative bacteria. *J. Gen. Microbiol.* **21**:ii-iii.
- HAWARD, A. C., W. G. C. FORSYTH, AND J. B. ROBERTS. 1959. Synthesis and breakdown of poly- $\beta$ -hydroxybutyric acid by bacteria. *J. Gen. Microbiol.* **20**:510-518.

- KALLIO, R. E., AND A. A. HARRINGTON. 1960. Sudanophilic granules and lipid of *Pseudomonas methanica*. J. Bacteriol. **80**:321-324.
- LEMOIGNE, M. 1926. Produits de déshydratation et de polymérisation de l'acid  $\beta$ -oxybutyrique. Bull. soc. chim. biol. **8**:770-782.
- LEVINE, H. B., AND H. WOLOCHOW. 1960. Occurrence of poly- $\beta$ -hydroxybutyric acid in *Pseudomonas pseudomallei*. J. Bacteriol. **79**:395-396.
- MACRAE, R. M., AND J. F. WILKINSON. 1958. Poly- $\beta$ -hydroxybutyrate metabolism in washed suspensions of *Bacillus cereus* and *Bacillus megaterium*. J. Gen. Microbiol. **19**:210-222.
- MORRIS, M. B., AND J. B. ROBERTS. 1959. A group of pseudomonads able to synthesize poly- $\beta$ -hydroxybutyric acid. Nature **183**:1538-1539.
- SLEPECKY, R. A., AND J. H. LAW. 1960a. The role of poly- $\beta$ -hydroxybutyric acid in the sporulation of *Bacillus megaterium*. Bacteriol. Proc. **1960**:64.
- SLEPECKY, R. A., AND J. H. LAW. 1960b. A rapid spectrophotometric assay of  $\alpha$ ,  $\beta$ -unsaturated acids and  $\beta$ -hydroxy acids. Anal. Chem. **32**:1697-1699.
- STANIER, R. Y., M. DOUDOROFF, R. KUNISAWA, AND R. CONTOPOULOU. 1959. The role of organic substrates in bacterial photosynthesis. Proc. Natl. Acad. Sci. U. S. **45**:1246-1260.
- WEIBULL, C. 1953. Characterization of the protoplasmic constituents of *Bacillus megaterium*. J. Bacteriol. **66**:696-702.
- WILKINSON, J. F. 1959. The problem of energy-storage compounds in bacteria. Exptl. Cell Research (Suppl.) **7**:111-130.
- WILLIAMSON, D. H., AND J. F. WILKINSON. 1958. The isolation and estimation of poly- $\beta$ -hydroxybutyrate inclusions of *Bacillus* species. J. Gen. Microbiol. **19**:198-209.